Interactions of cell division protein FtsZ with large and small molecules

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CHAPTER 4

The effects of manganese on the interaction of sporulation protein SpoIIE with itself and cell division protein FtsZ

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Experiments presented in Figures 1, 2, 4, 5 and 6 were performed by E. Cendrowicz.

ABSTRACT

Sporulation is an adaptive process undertaken by *Bacillus subtilis* and its relatives under starvation conditions, that results in the formation of a dormant cell called a spore. It begins with the formation of a polar septum, which divides the cell into two unequal-sized cells, a larger mother cell and a smaller forespore. After polar septum formation, a transcription specific factor, σ^F is activated in the forespore. Both events require a protein called SpoIIE. SpoIIE interacts directly with the cell division protein FtsZ and localizes together with FtsZ to the polar septa. The second function of SpoIIE is the activation of σ^F by dephosphorylating the anti-sigma factor antagonist SpoIIAA, a process depending on the cofactor Mn^{2+}. Here, we report our attempts to study the FtsZ--SpoIIE interaction *in vitro*. We demonstrate influence of divalent metals on the oligomerization of the cytoplasmic domain of SpoIIE. We investigate the influence of the SpoIIE cofactor, Mn^{2+}, on the oligomerization of FtsZ. Finally, we study direct interaction between FtsZ and SpoIIE and show clear interaction between these two proteins in the presence of GDP.

INTRODUCTION

In response to starvation conditions, *Bacillus subtilis* cells cease vegetative growth and initiate formation of a dormant cell type called a spore. The first clear step in this process is the formation of the asymmetrically positioned septum that divides the cell into two daughter cells of different size: the larger mother cell and the smaller forespore, that each receive one copy of the chromosome. Next, compartment specific transcription factors σ^F and σ^E are activated in the forespore and in the mother cell respectively. This activation event is critical because it initiates the rest of the sporulation developmental program in each daughter cell. Ultimately, the forespore becomes the spore and the mother cell lyses when the process is complete [1-5].

Upon entry into sporulation, the cell division protein FtsZ, that during vegetative growth drives the mid-cell division, switches its position to the polar sites of the cell. The switch is triggered by an integral membrane protein called SpoIIE [1,6-8]. At the onset of sporulation, SpoIIE co-localizes with FtsZ at mid-cell and both proteins redeploy to polar sites via a spiral-like intermediate [6]. Next, one of the polar Z-rings disassembles and the other one is converted into a division septum. SpoIIE contributes to the formation of the polar Z-rings but is not required for the process. It was shown that in *spoIIE* null mutant cells, FtsZ may still localize to the polar sites but the switch from medial to polar rings is delayed and the frequency of polar Z-ring formation decreased. After constriction of the polar Z-ring, FtsZ is released to the cytosol and SpoIIE remains associated with the polar septum and performs its second function, which involves its phosphatase domain [2]. It activates σ^F by dephosphorylating the anti-sigma factor antagonist SpoIIAA [3,9]. SpoIIE phosphatase is inactive in the pre-divisional cell and becomes active only after the asymmetric septum is formed [10]. Thus, it is thought that FtsZ may be involved in the activation of phosphatase of SpoIIE. SpoIIE is a transmembrane protein with a three-domain structure. The N-terminal domain (domain I) consists of 10 membrane-spanning segments, the central, poorly conserved domain (domain II) is involved in the oligomerization of SpoIIE and the interaction with FtsZ and the C-terminal domain (domain III) is structurally related to the PP2C (Protein phosphatase 2C), Mn^{2+}-dependent family of protein phosphatases (Fig. 1A) [11-14]. There is a clear structural

separation of the three domains of SpoIIE. It was shown that the C-terminal domain can independently function as a phosphatase *in vitro*, and that the central domain may interact with either FtsZ or itself, independent of the other two domains. However, several *in vivo* studies found that some mutations in the domain II influence the phosphatase activity of SpoIIE [10]. It was also shown that some mutations in domain III that impair the phosphatase activity also impair polar cell division [7].

The interaction between SpoIIE and FtsZ was previously shown *in vitro* [15]. However, details about the SpoIIE-FtsZ interaction and SpoIIE self-interaction are not known because of difficulties in the expression of the soluble cytoplasmic domain of SpoIIE [16]. In this work, we purified the soluble part of SpoIIE ($SpoIIE_{cyt}$) fused to maltose binding protein (MBP) and studied the interaction of SpoIIE with FtsZ and itself in more detail.

MATERIALS AND METHODS

PLASMID CONSTRUCTION

pMalC2x (New England Biolabs) was used to clone and purify strep-$SpoIIE_{cyt}$ as an N-terminal MBP-strep-$SpoIIE_{cyt}$ (Ms-$SpoIIE_{cyt}$) fusion (Fig. 1A). A 1503 bps fragment coding for *spoIIE*$_{cyt}$ was amplified by PCR from *B. subtilis* 168 template genomic DNA using a forward primer containing the strep-tag coding sequence (bold) (ek61 5′-**AGCGCTTGGCGTCACCCGCAGTTCGGTGGT**CCTCAATCTATTACGAGGAAAGTGG) [10] and a reverse primer containing a *Bam*HI site (underlined) (ek62 5′-GCGGATCCCATATATTCCCATCTTCGCCAGAAG). The PCR product was digested using *Bam*HI and ligated into pMalC2x linearized with *Xmn*I (blunt end)/*Bam*HI, resulting in plasmid pEK33.

PROTEIN EXPRESSION AND PURIFICATION

FtsZ was purified as described [17,18]. His-$EzrA_{cyt}$ was purified as described [19]. Ms-$SpoIIE_{cyt}$, MBP-SepF124 [20] or MBP were produced in *E. coli* BL21-RIL cells using the same method. Freshly transformed cells were grown overnight on

LB agar plates containing 100 µg/ml ampicillin and 50 µg/ml chloramphenicol. Liquid LB medium containing the same antibiotics was inoculated with a single colony from a fresh plate. An overnight culture was diluted 1:100 into fresh LB containing the same antibiotics and grown at 37°C until OD_{600}=0,7. Protein expression was induced by addition of 1 mM IPTG at 37°C for 3 hours. For purification, cells were resuspended in 50 mM Tris/ HCl pH=7.5, 300 mM KCl, 0.5 mM DTT and 0.1% Triton X-100 and disrupted by sonication. The cell lysate was clarified by centrifugation at 20 000 ×*g* for 20 min and the supernatant was applied onto amylose resin. The resin was washed with the same buffer without Triton X-100 and protein was eluted with 50 mM Tris/ HCl pH=7.5, 300 mM KCl, 0.5 mM DTT and 10 mM maltose. All three proteins were concentrated using Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore). The protein concentration was measured and absorption spectra were taken using a NanoDrop ND-1000 Spectrophotometer (ISOGEN Lifescience). An extinction coefficient (Ex=107,720) was determined using an ExPASy ProtParam tool using the Ms-SpoIIE$_{cyt}$ sequence.

To obtain strep-SpoIIE$_{cyt}$, the protein solution was mixed with an equal volume of 50 mM Hepes/ NaOH pH=7.5, 1M KCl, 0.5mM DTT, 1mM EDTA and 1% Triton X-100 to obtain final concentrations of 650 mM KCl and 0.5% Triton X-100. The MBP tag was cleaved overnight at 4°C using Factor Xa protease (New England Biolabs). Factor Xa was deactivated using Dansyl-glu-gly-arg-chloromethyl ketone and the protein mixture was applied onto HiLoad Superdex 16/600 gel filtration column and eluted with 50 mM Hepes/ NaOH, pH=7.5, 650 mM KCl, 0.5 mM DTT, 1 mM EDTA and 0.5% Triton X-100.

ICP-OES MEASUREMENT

Purified Ms-SpoIIE$_{cyt}$ from two independent purifications was lyophilized and analysed for the presence of calcium, iron, magnesium, manganese and zinc using inductively-coupled plasma optical emission spectroscopy on an Optima 7000DV ICP-OES (PerkinElmer) apparatus. The measurements were performed in duplicate.

LIGHT SCATTERING

The effects of various cations on the oligomerization of strep-SpoIIE$_{cyt}$ were monitored by 90° light scattering using an AMINCO-Bowman Series 2 fluorescence spectrometer. Strep-SpoIIE$_{cyt}$ or MBP (1.5 μM) were incubated in 50 mM MES/NaOH pH=6.5, 300 mM KCl buffer. After 60 seconds of incubation various chloride salts of divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+} or Co^{2+}, Ni^{2+}) were added to the solution and the light scattering signal was monitored for 1 hour.

The reversal of strep-SpoIIE$_{cyt}$ oligomerization by EDTA was done in a larger volume cuvette (1 ml) to allow stirring, and measurements were taken using a QuantaMaster™ spectrofluorometer controlled by the FelixGX program (Photon Technology International, Inc.). $MnCl_2$ (10 mM final concentration) was added to the sample after 3.5 min of incubation and EDTA (20 mM final concentration) was added after 10 minutes of incubation. As a dilution control, H_2O was added to the sample. The light scattering signal of protein without Mn^{2+} was measured as blank.

The effect of strep-SpoIIE$_{cyt}$ (1.5 μM) on the assembly of FtsZ (10 μM) was studied in 50 mM Tris/HCl, pH=7.5, 300 mM KCl and 10 mM $MgCl_2$. After 90 sec of measurement GTP or GDP (2 mM final concentration) was added to the sample. As controls, measurements were taken of strep-SpoIIE$_{cyt}$ and/or FtsZ in the presence or absence of nucleotides. All experiments were done at 30°C.

CRITICAL CONCENTRATION DETERMINATION

To study changes in critical concentration for oligomerization of Ms-SpoIIE$_{cyt}$ in the presence and absence of Mn^{2+}, fluorescence spectra of increasing concentration of Ms-SpoIIEcyt-Cy5 were acquired in the presence of Mn^{2+} or EDTA. Ms-SpoIIE$_{cyt}$ was purified from lysates by binding to a amylose resin as described above, with the inclusion of an on-column Cy5 labeling step. After washing column bound protein with buffer A (50 mM Tris/ HCl pH=7.5, 300 mM KCl, 0.5 mM DTT), the resin with Ms-SpoIIE$_{cyt}$ was mixed with fluorescent label Cy5 (GE Healthcare) and incubated for 1 hour at 4°C. Excess label was washed away using the same buffer and labeled protein was eluted with buffer A sup-

plemented with 10 mM maltose. For the measurement equal volumes (12 µl containing 0.23 µM Ms-SpoIIE$_{cyt}$-Cy5) of Ms-SpoIIE$_{cyt}$-Cy5 was titrated to a buffer containing 50 mM Tris/HCl, 300 mM KCl, 1 mM EDTA (for the measurement without manganese) or 10 mM $MnCl_2$ (for the measurement with manganese). The measurements were taken immediately after addition of Ms-SpoIIE$_{cyt}$ at 30°C. Fluorescence was excited at 633 nm and emission spectra (640-685 nm) were acquired in a QuantaMaster™ spectrofluorometer controlled by the Felix-GX program (Photon Technology International, Inc.).

SEDIMENTATION ASSAY

FtsZ (10 µM) was mixed with Ms-SpoIIE$_{cyt}$ (10 µM) in polymerization buffer (50 mM Hepes/ NaOH, pH=7.5, 50 mM KCl, 10 mM $MgCl_2$). After incubation for 15 min at 30°C, an equal volume of GTP or GDP was added to the samples (final concentration 2 mM). The samples were incubated for another 15 min at 30°C and centrifuged at 186 000 ×*g* for 15 min at 20°C. Pellet and supernatant fractions were analyzed by SDS-PAGE as described [17]. Two controls were used in this assay. MBP-SepF124 (10 µM), a MBP fusion of SepF mutant that does not interact with FtsZ [20] was used in place of Ms-SpoIIE$_{cyt}$ to see whether MBP is not a cause of increase in sedimentation of FtsZ. To check whether the interaction between Ms-SpoIIE$_{cyt}$ and FtsZ is specific, His-EzrAcyt (10 µM) was used in the assay instead of FtsZ.

For the sedimentation assay of Ms-SpoIIE$_{cyt}$ alone, Ms-SpoIIE$_{cyt}$ was preincubated for 20 min in 100 µl of polymerization buffer (50 mM MES/ NaOH, pH=6.5, 50 mM KCl) supplemented with 1 mM EDTA. 30 µl of the sample was collected for further analysis. After that, the sample was supplemented with 5 mM $MnCl_2$ and incubated for another 30 min. After the incubation time, 30 µl sample was collected. The rest of the sample was supplemented with 10 mM EDTA and incubated for another 40 min. All the samples collected after each incubation time were collected, spun down at 186 000 ×*g* and the supernatants and pellets were separated for analysis by SDS-PAGE.

ELECTRON MICROSCOPY

To visualize Ms-SpoIIE$_{cyt}$ using Transmission Electron Microscopy (TEM), 10 µM Ms-SpoIIE$_{cyt}$ was prepared in 50 mM Hepes/ NaOH, pH=7.5, 50 mM KCl.

To visualize FtsZ in the presence or absence of Ms-SpoIIE$_{cyt}$, 10 µM FtsZ with or without 5 µM Ms-SpoIIE$_{cyt}$ was prepared in polymerization buffer: 50 mM Tris/ HCl, pH=7.5, 300 mM KCl supplemented with 1 mM EDTA or 10 mM $MgCl_2$ or 10 mM $MnCl_2$. After 5 min of incubation 2 mM GTP was added to the mixture. 4 µl of each sample was collected in appropriate time points and applied onto glow discharged carbon grids and grids were prepared as described [17]. The grids were examined in a Philips CM120 electron microscope equipped with a LaB_6 filament operating at 120 kV. Images were recorded with a Gatan 4000 SP 4 K slow-scan CCD camera at magnifications 36,400 × (for FtsZ ± Ms-SpoIIE$_{cyt}$) or 45,500 × (for Ms-SpoIIE$_{cyt}$ structures alone).

FLUORESCENCE MICROSCOPY

An overnight culture of *B. subtilis* strain 4055 [19] grown in CH medium [21] was diluted into fresh CH to an OD of 0.1. Cells were grown at 37°C until OD of 0.7. At this point, 2 samples of 5 ml were taken and cells were collected and washed 2 times with the same volume of CH with (spo+) or without (spo-) manganese. After the washing steps, spo+ and spo- cells were resuspended in 100 µl of CH with and without manganese, respectively. Sporulation medium (with or without manganese) was added up to the volume of 5 ml in the presence of 0.02 mM of IPTG. Cells were allowed to sporulate at 37°C by continuing the incubation. Every hour, a 500 µl sample was taken and cells were harvested and resuspended in 20-50 µl of PBS before being mounted on an agarose pad prior to microscopy. FtsZ-eYFP was visualized as described in [19] and cells were scored according to their Z-ring localization.

GTPASE ASSAY

The GTP hydrolysis rate determination was performed as described in [17]. Ms-SpoIIE$_{cyt}$ was pre-incubated with 1 mM EDTA to remove metal bound to the

protein during purification to obtain Ms-SpoIIE$_{cyt}$-apo. Ms-SpoIIE$_{cyt}$-Fe – metal bound during purification, Ms-SpoIIE$_{cyt}$-apo or MBP were incubated with FtsZ in polymerization buffer: 50 mM Hepes/ NaOH, pH=7.5, 300 mM KCl, 1 mM EDTA. $MgCl_2$ was added in the same time as GTP to avoid oligomerization of Ms-SpoIIE$_{cyt}$ before polymerization of FtsZ.

RESULTS

SPOIIE CO-PURIFIES WITH BOUND METAL IONS

The 501 residue C-terminal fragment of SpoIIE (amino acids 326-827) was N-terminally fused to maltose binding protein (MBP) via a short strep-tag. The Ms-SpoIIE$_{cyt}$ protein was purified with a high yield (~ 40 mg/1L culture). The absorption spectrum of purified Ms-SpoIIE$_{cyt}$ showed a broad peak in the 420 nm region, similar to those of iron/manganese-protein complexes [22], suggesting that Ms-SpoIIE$_{cyt}$ was purified in a metal bound form. The absorption spectrum of purified MBP-SepF124 (which will serve as a control protein for some assays) did not show similar peak at this region. The C-terminal domain of SpoIIE belongs to the Mn^{2+}- dependent phosphatase 2C family of phosphatases [14]. We conducted an ICP-OEC measurement of lyophilized Ms-SpoIIE$_{cyt}$. The measurement revealed Fe bound to the sample in the ratio close to 2:1 (Fig. 1B). As iron is very similar to manganese, we think that it simply replaced manganese in its active site during folding in the heterologous host. Our results show 2 metal ions bound per one SpoIIE molecule. The analysis also revealed small amounts of Ca and Mg bound to the protein but manganese levels were below the detection limit (Fig. 1B).

It was possible to replace iron with manganese by incubation of protein with EDTA followed by incubation with excess of $MnCl_2$. After incubation with EDTA, the absorption peak at 420 nm disappeared and appeared again when the protein was incubated with $MnCl_2$. However, the spectrum had a lower peak compared to the spectrum of Ms-SpoIIE$_{cyt}$-Fe obtained from the purified sample. We do not know if the metal binding site was again fully occupied by manganese.

Strep-SpoIIE$_{cyt}$ was further purified using gel filtration after cleavage of the MBP tag. The purification was performed in the presence of 650 mM KCl and 0.5% TritonX-100 as the protein was more stable under these conditions. Strep-SpoIIE$_{cyt}$ was eluted in several fractions corresponding to a range of molecular weights between 60 and 500 kDa, indicating the presence of oligomers of up to 9-10 subunits. This result is in agreement with an earlier study that describes oligomerization of SpoIIE [15]. As the purification buffer was not compatible with visualization of the oligomers using elactron microscopy, we diluted Ms-SpoIIE$_{cyt}$ into a 50 mM Hepes/ NaOH pH=7.5, 50 mM KCl buffer to a final concentration of 10 µM. We found two different structural species on the grid, rod shaped protein oligomers and circular structures (Fig. 1C). The circular structures may represent rods which bound perpendicular to the carbon grid (top view of Ms-SpoIIE$_{cyt}$ oligomers) whereas rods represent a side view of the Ms-SpoIIE$_{cyt}$ oligomers (Fig. 1C).

METAL BINDING ENHANCES OLIGOMERIZATION OF STREP-SPOIIE$_{CYT}$

It was shown before that domain II (amino acids 321-567) is involved in the oligomerization of SpoIIE (Fig. 1A) [15]. We wanted to study the influence of metal ions on strep-SpoIIE$_{cyt}$ oligomerization and therefore performed light scattering measurement of strep-SpoIIE$_{cyt}$ in the presence of its cofactor Mn^{2+} and other divalent cations. Strep-SpoIIE showed an increase in light scattering signal upon binding to its cofactor. We wanted to see whether Fe, which was co-purified with Ms-SpoIIE$_{cyt}$, has a similar effect on strep-SpoIIE$_{cyt}$. However, Fe^{2+} becomes oxidized very easily in solution and the analysis in the presence of this metal was not possible even when reducing agents like DTT or ascorbate were added to the buffer. Therefore we tested other divalent metal ions, which are commonly used as cofactors by biological systems, and thus may easily bind to the same site on SpoIIE as manganese. All the metals used had a significant influence on the oligomerization of strep-SpoIIE$_{cyt}$ (Fig. 2A). It is known that binding preferences of metals to some proteins do not always correspond to their metal requirements [23]. This feature was also observed for strep-SpoIIE$_{cyt}$. Light scattering signal was the highest in the presence of the strongest binding metal Zn^{2+} or Ni^{2+} and the lowest in the presence of Mg^{2+}

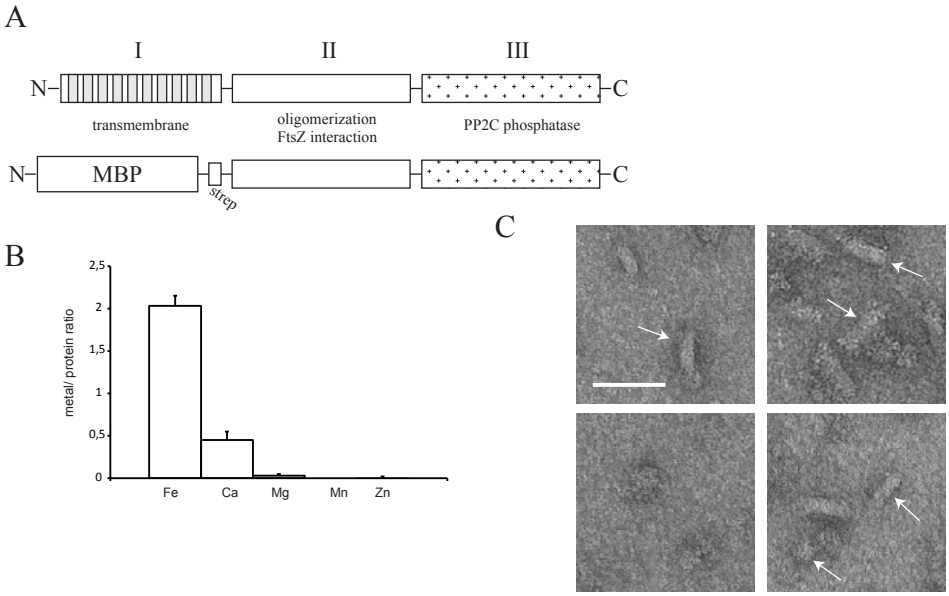

Figure 1. (A) The putative three domain structure of SpoIIE (top) and a schematic of the SpoIIE construct used in this study (bottom), (B) ICP-OES measurement of lyophilized Ms-SpoIIEcyt revealed saturation of Ms-SpoIIEcyt with Fe and small amounts of other divalent cations but not Mn. (B) Electron microscopy of Ms-SpoIIEcyt oligomers after co-purification with Fe. Arrows point to rod and circular structures of Ms-SpoIIEcyt. Scale bar: 50 nm.

(Fig. 2A), according to Irving-Williams series [23]. Like Fe, Cu was excluded from the measurement due to its instability/high reactivity with buffer components. To prove that the increase in light scattering signal is not due to reaction of metals with some buffer components, we used MBP as a control and did not see an increase in light scattering signal (Fig. 2B). Metal-dependent oligomerization of Ms-SpoIIE$_{cyt}$ was in agreement with the observation that Ms-SpoIIE$_{cyt}$ was always recovered in the pellet fraction when used in FtsZ assays, which are performed in the presence of Mg^{2+} (Fig. 5B, C).

To study the critical concentration of Ms-SpoIIE$_{cyt}$ necessary to form oligomers we labeled Ms-SpoIIE$_{cyt}$ with the fluorescent dye Cy5. Labeled protein was titrated into a buffer supplemented with either EDTA or $MnCl_2$. The fluorescence signal continuously increased upon addition of Ms-SpoIIE$_{cyt}$-Cy5 until it reached the concentration at which signal was quenched by oligomerization of Ms-SpoIIE$_{cyt}$-Cy5 and did not further increase (Fig. 2C, D). The emission spec-

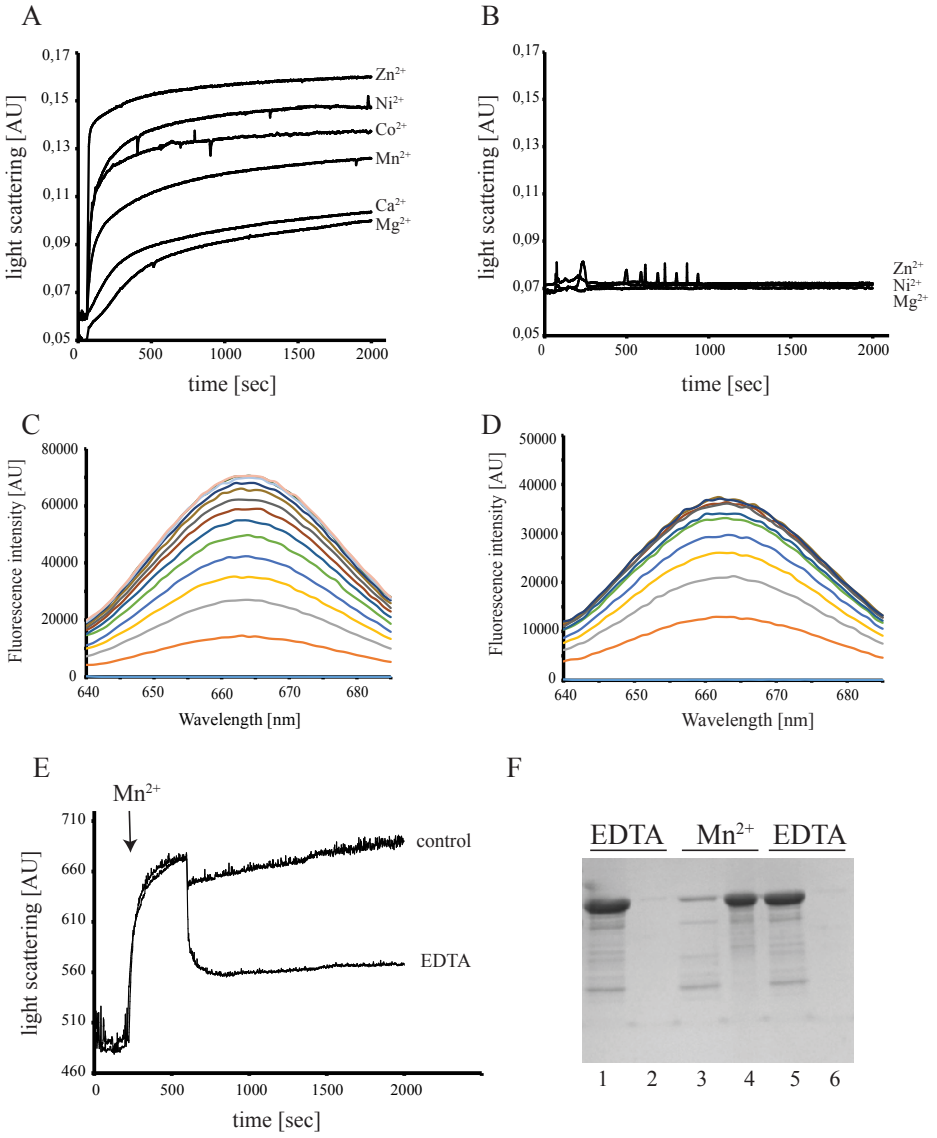

Figure 2. Binding to divalent cations enhances oligomerization of strep-SpoIIEcyt. (A, B) Light scattering measurement of 1,5 μM strep-SpoIIEcyt (A) and MBP (B) in the presence of 10 mM of divalent cations. (C, D) Fluorescence emission spectra of strep-SpoIIEcyt-Cy5 titrated into a buffer in the absence (C) and presence (D) of Mn^{2+}. (E) Light scattering signal of strep-SpoIIEcyt in the presence of 10 mM Mn^{2+}, followed by incubation with 20 mM EDTA or H2O (control). (F) SDS-PAGE of the sedimentation of Ms-SpoIIEcyt experiment. Ms-SpoIIEcyt was pre-incubated with 1 mM EDTA (lanes 1 and 2), followed by the incubation with 5 mM $MnCl_2$ (lanes 3 and 4), followed by the incubation with 10 mM EDTA (lanes 5 and 6). Odd numbers below the image represent supernatant fractions and even numbers represent pellet fractions.

tra in the presence of metal (Fig. 2D) gave significantly lower signals compering to the sample with EDTA (Fig. 2C) possibly due to binding of the protein to metal ions or formation of dimers. However, our goal was not to compare the spectra intensities between two experiments but to find the concentration in which fluorescence did not increase within one experiment. We noticed that the critical concentration of Ms-SpoIIE$_{cyt}$-Cy5 was higher in the absence of Mn^{2+} (~2.1 μM) compering to the sample in the presence of Mn^{2+} (1.4 μM). In the absence of divalent cation strep-SpoIIE$_{cyt}$ is also able to form oligomers, which is in agreement with the oligomerization observed during purification and previous results [15]. However, these oligomers are possibly smaller and not as stable as oligomers formed by strep-SpoIIE$_{cyt}$ in the presence of Mn^{2+}.

MANGANESE-DEPENDENT OLIGOMERIZATION OF MS-SPOIIE$_{CYT}$ AND STREP-SPOIIE$_{CYT}$ IS REVERSIBLE

We wanted to see whether the observed increase in light scattering is due to the oligomerization rather than aggregation of strep-SpoIIE$_{cyt}$. Therefore, we performed light scattering and sedimentation experiments, in which addition of manganese to the sample was followed by incubation with EDTA. As before, the light scattering signal changed immediately after addition of $MnCl_2$ to strep-SpoIIE$_{cyt}$. Subsequent addition of EDTA to the sample caused an immediate decrease to the light scattering signal (Fig. 2E), which was not due to dilution or buffer effects as evidenced by the addition of an equal volume of buffer. The light scattering signal did not fully return to the background signal, probably because some manganese is trapped between monomers and cannot be released completely.

Next, Ms-SpoIIE$_{cyt}$ was incubated with EDTA to remove all metals bound to the protein during purification. Subsequently, the protein was incubated with Mn^{2+}, followed by additional incubation with EDTA. Samples of all three stages were centrifuged and supernatants and pellets were analyzed by SDS-PAGE. The initial incubation with EDTA resulted in Ms-SpoIIE$_{cyt}$ in the supernatant, which indicates that the protein is in monomeric or forms small oligomers (Fig. 2F, lanes 1 and 2). After Mn^{2+} addition, oligomers of Ms-SpoIIE$_{cyt}$ were recovered in the pellet fraction (Fig. 2F, lane 4). This oligomerization could be re-

versed by incubation with EDTA, after which MBP-SpoIIE$_{cyt}$ was again present in the supernatant (Fig. 2F, lane 5). Combined, the light scattering and sedimentation experiments show that the metal-dependent oligomerization of SpoIIE is reversible and thus not caused by protein aggregation.

THE ABSENCE OF MANGANESE DELAYS POLAR Z-RING FORMATION *IN VIVO*

It has been known for a long time that the sporulation process is strongly dependent on the presence of manganese, but so far this dependence has been attributed to the role of manganese as a cofactor in the phosphatase activity of SpoIIE, which is required for the activiation of the forespore specific transcription factor σ^F [1]. Because the metal-dependent oligomerization of SpoIIE may be required for the function of SpoIIE in polar septation, we studied FtsZ-eYFP localization in sporulating *B. subtilis* cells in the presence and absence of manganese. In the absence of manganese, relocation of the Z-ring from mid-cell to the cell poles is delayed compared to samples supplemented with Mn^{2+}. We noticed that cells without Mn^{2+} form more mid-cell rings and significantly less polar rings compared to the cells with Mn^{2+} (Fig. 3). It is clear that asymmetric septum formation is affected in the absence of manganese. Interestingly, the delay and decrease in the formation of asymmetric Z-rings is also observed in a *spoIIE* null mutant [8], suggesting that in the absence of manganese SpoIIE does not interact with FtsZ or cannot support FtsZ relocation to the cell pole. However, we cannot fully exclude indirect effects such as a decrease in expression of SpoIIE in the absence of its cofactor, or another mechanism which is affected by the absence of Mn^{2+}.

FTSZ POLYMERS ARE STABLE AND DO NOT DEPOLYMERIZE IN THE PRESENCE OF MN^{2+}

Next, we wanted to study the interaction between strep-SpoIIE$_{cyt}$ and FtsZ. Because the activities of both proteins depend on divalent cations, we wanted to choose the best conditions to study the FtsZ-strep-SpoIIE interactions. The presence of Mg^{2+}, which is a FtsZ cofactor, clearly influences the oligomeric

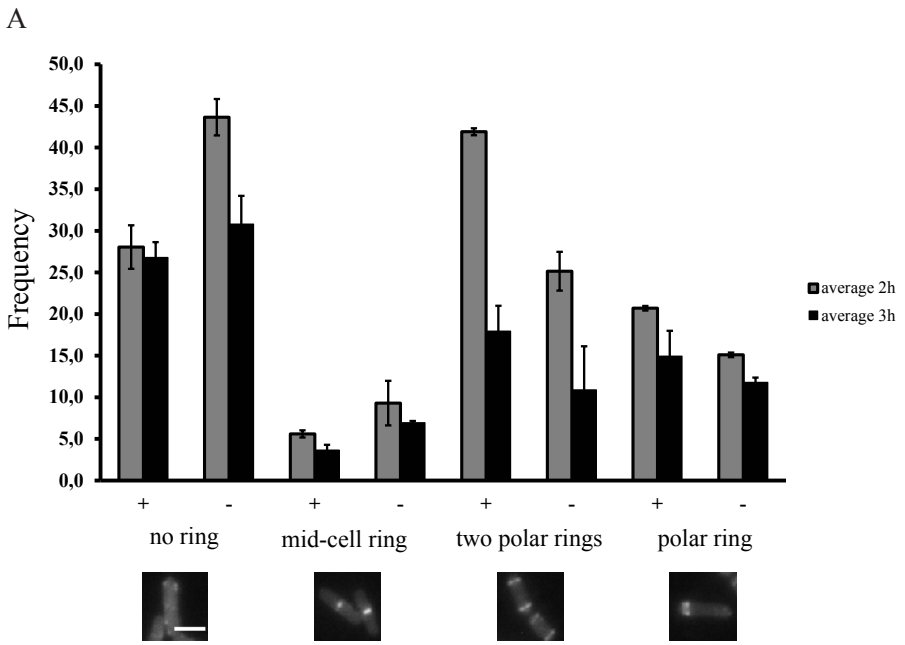

Figure 3. The absence of Mn^{2+} in the sporulation medium delays the asymmetric Z-ring formation. Representation of counting of sporulating *B. subtilis cells* in the presence (+) and absence (-) of Mn^{2+}. Four different populations were found: cells without any Z-ring in the cell (no ring), cells with clearly formed Z-ring in the middle of the rod (mid-cell ring), cells with two rings assembled at the cell poles (two polar rings) and cells with only one polar ring (polar ring). Below the graph, representative pictures of each of the population are present. More than 100 cells were counted per each time point.

state of strep-$SpoIIE_{cyt}$ (Fig. 2A). To study whether the strep-$SpoIIE_{cyt}$ cofactor Mn^{2+} has any influence on the assembly of FtsZ, light scattering and EM were used. FtsZ assembly after addition of GTP in the presence of Mn^{2+} is similar to the assembly of FtsZ in the presence of Mg^{2+} (Fig. 4A). However, after prolonged incubation times (>30 min), the light scattering signal began to increase and after ~45 min reached the detection limit (Fig. 4B), with FtsZ precipitating out of the solution. To identify the cause of the precipitation the sample was analyzed using EM. In the presence of Mn^{2+}, FtsZ forms stable polymers, which are not able to depolymerize even after 90 min of incubation (Fig. 4F), which is probably caused by the block in GTP hydrolysis. In the absence of polymer dynamics, polymers continuously assemble into long thick structures, which condense and precipitate from the solution. This is similar to FtsZ-SepF tubules,

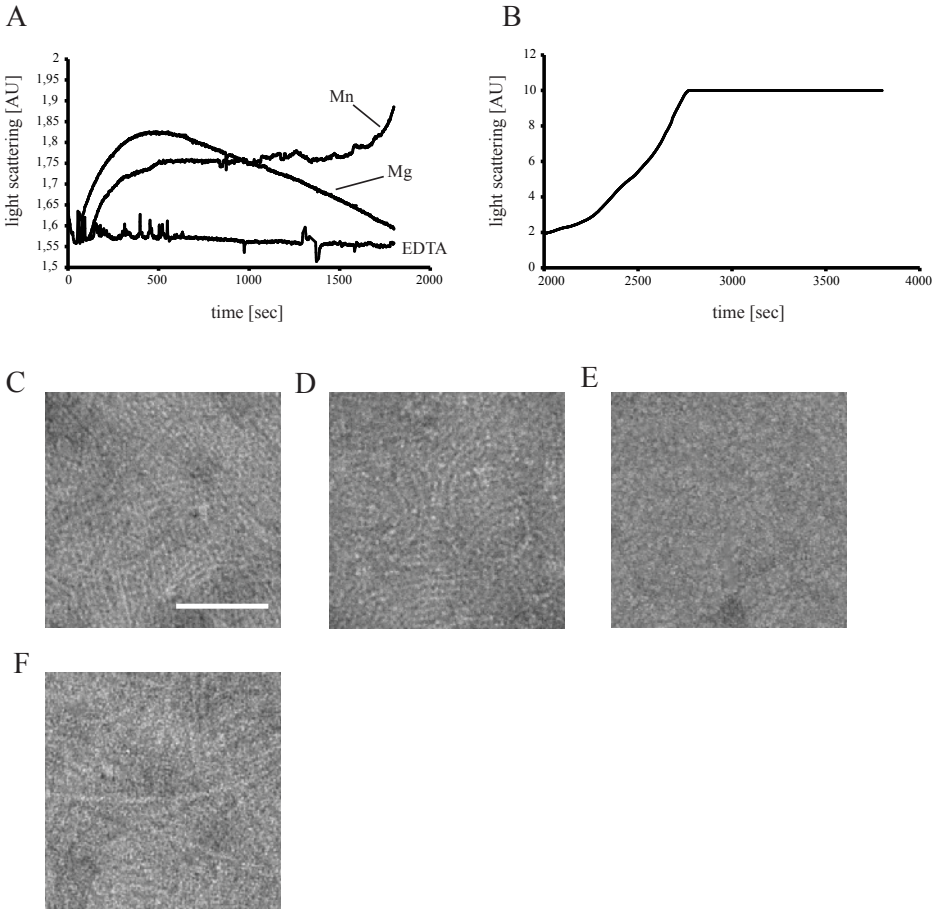

Figure 4. (A) Light scattering signal of FtsZ in the presence of 2 mM GTP and 10 mM of divalent cations (Mn^{2+}, Mg^{2+}) or 1 mM EDTA. (B) continuation of the signal from the sample with Mn^{2+}. (C, D, E) Electron microscopy of FtsZ polymers assembled in the presence of 2 mM GTP and either Mn^{2+} (C), EDTA (D) or Mg^{2+} (E) after 30 min of incubation. (D) Representative picture of FtsZ polymers assembled in the presence of 2 mM GTP and Mn^{2+} after 90 min of incubation.

which are also very big structures and precipitate out of the solution when high GTP concentration is used (not shown).

FtsZ samples were also visualized after 30 min of incubation. In the presence of Mg^{2+}, FtsZ polymers were almost not visible or very short (Fig. 4E), which means they had disassembled in agreement with the decrease in light scattering signal (Fig. 4A). In the presence of Mn^{2+}, FtsZ polymers are still long and form large bundles (Fig. 4C). Polymers formed in the absence of metal

were still present after 90 min of incubation (Fig. 4D) but were not as dense as polymers formed in the presence of Mn^{2+} (Fig. 4C). Because the presence of Mn^{2+} has such a significant effect on the FtsZ properties, we decided to exclude it from the FtsZ-strep-SpoIIE$_{cyt}$ interaction studies.

DIRECT INTERACTION BETWEEN FTSZ AND STREP-SPOIIE$_{CYT}$ OCCURS IN THE PRESENCE OF GDP

A direct interaction between FtsZ and SpoIIE has been shown by Lucet *et al*. using several techniques like gel filtration, non-denaturing PAGE and pull-down experiments [15]. Further attempts to do study the FtsZ-SpoIIE interaction *in vitro* in more detail faltered because of difficulties in the purification of SpoIIE [16]. To study the effect of strep-SpoIIE on FtsZ, the light scattering experiment of FtsZ in the presence of strep-SpoIIE$_{cyt}$ without manganese was initially performed. The experiment was performed in a buffer with high salt concentration as strep-SpoIIE$_{cyt}$ is more stable under those conditions. Strep-SpoIIE$_{cyt}$ enhanced the light scattering of FtsZ both in the presence of GTP and GDP (Fig. 5A). Notably, the scattering increased to higher levels than the scattering signals of the individual proteins combined. Strep-SpoIIE$_{cyt}$ alone gave a small light scattering signal, because of the presence of $MgCl_2$ in the sample, whereas the light scattering signal of FtsZ alone with GTP was very low because lateral interactions between FtsZ protofilaments are diminished in the buffer conditions used [17,24]. The increase in light scattering signal of FtsZ in the presence of strep-SpoIIE$_{cyt}$ had a significant lag phase, although assembly of FtsZ under these conditions should be immediate. To confirm immediate polymerization, FtsZ was polymerized in the same conditions but using a different signal amplification (Fig. 5A, FtsZ enhanced). Intriguingly, the increase of the signal of FtsZ in the presence of strep-SpoIIE$_{cyt}$ is noticeable in the moment that FtsZ polymers have started to disassemble.

In our hands, cleavage of the MBP tag from Ms-SpoIIE$_{cyt}$ resulted in an unstable protein that could not be obtained in high concentrations. Therefore, subsequent experiments with FtsZ were performed using Ms-SpoIIE$_{cyt}$. In the presence of GDP, FtsZ does not sediment, but when Ms-SpoIIE$_{cyt}$ is present significant amounts of FtsZ can be found in the pellet (Fig. 5B). This was in contrast to the sample with GTP, as the amount of FtsZ pelleted did not clearly increase

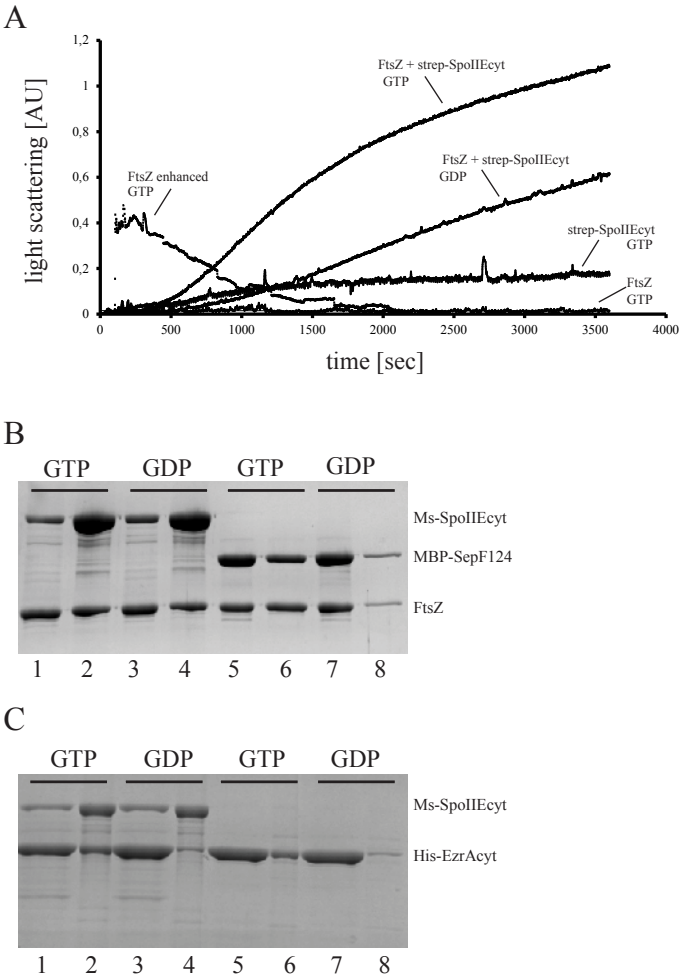

Figure 5. Strep-SpoIIEcyt interacts directly with FtsZ in the GDP form. (A) Light scattering measurement of either FtsZ or strep-SpoIIEcyt alone in the presence of GTP (FtsZ GTP or strep-SpoIIEcyt GTP respectively), in the presence of GTP and strep-SpoIIEcyt (FtsZ+strep-SpoIIEcyt GTP), in the presence of GDP and strep-SpoIIEcyt (FtsZ+strep-SpoIIEcyt GDP). Light scattering signal of FtsZ alone in the presence of GTP using enhanced light detection to show assembly/ disassembly path (FtsZ enhanced GTP). (B) Sedimentation assay of FtsZ in the presence of either Ms-SpoIIEcyt or MBP-SepF124 and in the presence of either GTP or GDP. (C) Sedimentation of His-EzrAcyt alone or in the presence of Ms-SpoIIEcyt with GTP or GDP. Odd numbers below the image represent supernatant fractions and even numbers represent pellet fractions.

in the presence of Ms-SpoIIE$_{cyt}$. Control experiments with MBP-SepF124 (which does not interact with FtsZ) showed that FtsZ sedimentation in the presence of GDP is not due to the presence of the MBP tag (Fig 5A). Another experiment

was performed with the control cell division protein His-EzrA$_{cyt}$. We noticed that Ms-SpoIIE$_{cyt}$ did not increase sedimentation of His-EzrA$_{cyt}$ in the presence of either GTP or GDP (Fig. 5C). Thus, the sedimentation of FtsZ in the presence of GDP and MBP-strep-SpoIIE$_{cyt}$ is specific.

Taking together the light scattering and sedimentation results, it is tempting to speculate that strep-SpoIIE$_{cyt}$ interacts with, and stabilizes, FtsZ in the GDP form. We do not think that FtsZ-GDP in the presence of Ms-SpoIIE$_{cyt}$ form higher order structures beyond FtsZ protofilaments (see below). The reason why FtsZ was recovered in the pellet fraction is that Ms-SpoIIE$_{cyt}$ is always present in the pellet after centrifugation in the presence of divalent cation. Therefore, Ms-SpoIIE$_{cyt}$ pulls FtsZ monomers/ short polymers to the pellet as a result of simple interaction. Also, the increase in the light scattering signal may be an effect of accumulation of FtsZ monomers/ short polymers on the surface of the strep-SpoIIE$_{cyt}$ oligomers, which together form bigger structures which scatter more light compared to strep-SpoIIE$_{cyt}$ alone (Fig. 5A).

INTERACTION BETWEEN MS-SPOIIE$_{CYT}$ AND FTSZ-GTP IS NOT DETECTABLE IN OUR ASSAYS

We did not find any significant increase in the sedimentation of FtsZ when both GTP and Ms-SpoIIE$_{cyt}$ were present in the sample. However, the sedimentation assay is not very sensitive and it is possible that FtsZ and Ms-SpoIIE$_{cyt}$ interact but that structures formed by these proteins together are not enough big to be spun down in the centrifuge. To further investigate the interactions in the presence of GTP we studied the GTPase activity of FtsZ in the presence of Ms-SpoIIE$_{cyt}$-apo (Ms-SpoIIE$_{cyt}$ preincubated with EDTA) and Ms-SpoIIE$_{cyt}$-Fe (Ms-SpoIIE$_{cyt}$ in the presence of Fe bound during expression of the protein). The GTPase activity of FtsZ does not significantly change in the presence of Ms-SpoIIE$_{cyt}$ when the ratio FtsZ:Ms-SpoIIE$_{cyt}$ is 2:1 (the small increase observed is close to the error of the experiment, (Fig 6A). We also decided to study FtsZ:Ms-SpoIIE$_{cyt}$ interactions using EM. We checked the assembly of FtsZ-GTP in the presence of Ms-SpoIIE$_{cyt}$ and did not find any significant difference in the filaments formed other that a mixture of FtsZ filaments next to Ms-SpoIIE$_{cyt}$ structures (Fig 6B, C, D). Therefore we conclude that Ms-SpoIIE$_{cyt}$ does not in-

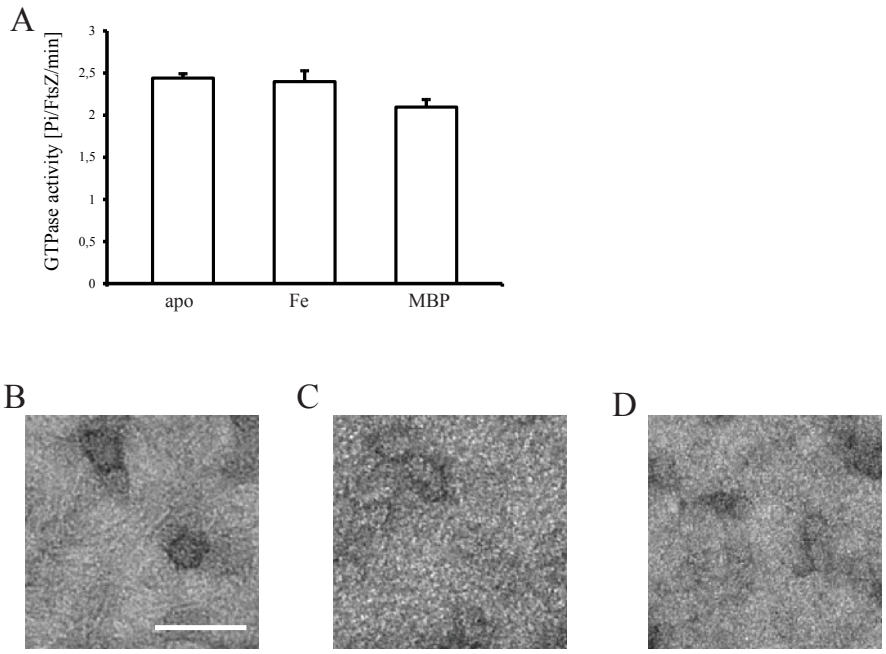

Figure 6. Ms-SpoIIEcyt does not interact with FtsZ in the presence of GTP. (A) GTPase activity of FtsZ in the presence of either Ms-SpoIIEcyt-apo, without intrinsic metal (apo), Ms-SpoIIEcyt-Fe, with Fe ion bound from the purification (Fe) or MBP. (B, C, D) Representative images of Electron microscopy experiments with FtsZ and Ms-SpoIIEcyt in the presence of 2 mM GTP and either Mn^{2+} (B), EDTA (C) or Mg^{2+} (D) after 20 min of incubation.

teract with FtsZ-GTP. When strep-SpoIIE$_{cyt}$ oligomerizes, the MBP tag, which is 40 kDa (almost half the size of the entire protein) might cover the binding region for FtsZ. It is possible that FtsZ polymers are too big to enter the binding site of strep-SpoIIE$_{cyt}$ but FtsZ monomers or short polymers formed in the presence of GDP are able to interact with strep-SpoIIE$_{cyt}$. Another possibility is that Ms-SpoIIE$_{cyt}$ prefers to interact with FtsZ-GDP as was already shown for some other FtsZ partners like MinC [25].

DISCUSSION

SpoIIE is a bifunctional protein involved in asymmetric septum formation and activation of the forespore compartment-specific transcription factor σ^F [1]. We

purified the entire cytoplasmic portion of SpoIIE fused to MBP via a short strep-tag. This cytoplasmic portion consists of two domains that carry out the functions of SpoIIE: Domain II is involved in oligomerization and interaction with FtsZ [15] and domain III is the phosphatase (Fig. 1A), that dephosphorylates and activates SpoIIAA-P, which in turn can induce the release of σ^F which is complexed with SpoIIAB. Dephosphorylation of SpoIIAA is an enzymatic process which can be run independently by domain III and is dependent on the presence of the cofactor, Mn^{2+} [14]. Our analysis revealed that Ms-SpoIIE$_{cyt}$ binds two metal ions and that metal binding is involved in the reversible oligomerization of SpoIIE$_{cyt}$. Because domain III was shown to be monomeric [14,15], we hypothesize that binding of Mn^{2+} to domain III induces a conformational change which resulted in oligomerization of domain II of SpoIIE, or that there is an extra metal binding site on domain II that is involved in oligomerization. Oligomerization was also induced by other divalent cations, which is not surprising as the binding specificity of enzymes for metal ions is quite low [26]. In the crystal structure of the phosphatase domain of SpoIIE, only a single manganese ion was found in the active site, which represented a fundamental difference between SpoIIE and the other type PP2C phosphatases, ex. human PP2Cα phosphatase of which active site binds two metal ions [14]. The difference between our data and the previous work could be because in the work of Ledikov *et al.* Tris and Hepes buffers were used for incubation of SpoIIE with Mn^{2+} [14]. As Tris has high affinity for divalent cations and Hepes clearly reacted with Mn^{2+} in our assays, we think the choice of buffer could be the cause of the low metal occupancy in the active site. It is also possible that domain II of SpoIIE is necessary for the stable metal binding by domain III. The other explanation is that the second metal can easily bind to SpoIIE during folding while it is not easy to introduce this metal into the protein structure while the protein is already folded, which was also observed in our analysis of the absorption spectrum of Ms-SpoIIE$_{cyt}$ after incubation with $MnCl_2$. Our data clearly demonstrate that domain II and III are not fully independent and may influence each other's activity, which is in agreement with the previous *in vivo* findings, where mutations in either of the domains had an influence on the activity of the other domains *in vivo* [7,10].

Manganese is important for the oligomerization and activity of SpoIIE but it has been known for a long time that it is also necessary for sporulation of

B. subtilis. It is known that Mn^{2+} is a cofactor for many enzymes involved in sporulation but it is not known at which stage is sporulation blocked in the absence of Mn^{2+}. Because of the role of SpoIIE in asymmetric septation, we decided to study localization of FtsZ-eYFP during sporulation in the presence and absence of Mn^{2+}. We noticed that in the absence of manganese, formation of the asymmetric septum is delayed and less polar Z-rings are formed compared to cells sporulating in the presence of Mn^{2+}. Only two proteins are known to be involved in the switch from medial to polar Z-ring, early sporulation-specific transcriptional factor Spo0A and SpoIIE. A mutation in the *spo0A* gene completely blocks sporulation at stage 0 before asymmetric septation occurs. Thus, it is unlikely that the lack of manganese influences Spo0A as it would completely block relocation of the Z-ring [8]. Localization of FtsZ in the absence of Mn^{2+} during sporulation resembles the situation in *spoIIE* mutant cells. It has been shown previously that deletion of the *spoIIE* gene affects formation of asymmetric Z-rings but does not prevent it [8]. Therefore, we conclude that the absence of manganese influences relocation of the Z-ring in a SpoIIE--dependent manner. However, we do not know whether our observations are the secondary effect of lower expression or misfolding of SpoIIE in the absence of Mn^{2+} or another mechanism which involves direct interactions between these two proteins. Because of lack of SpoIIE antibodies we could not check the levels of expression of SpoIIE in cells sporulating with and without manganese.

Our further analysis focused on the direct interaction between FtsZ and $SpoIIE_{cyt}$. Until now only two works studied the direct interaction between FtsZ and SpoIIE *in vitro*. Lucet *et al*. showed a clear direct interaction between domain II of SpoIIE and FtsZ using gel filtration [15]. A study by Rawlings *et al*. could not confirm these findings [16]. Lucet *et al*. purified a SpoIIE fragment containing amino acids 326-827, whereas Rawlings *et al*. used two SpoIIE fragments starting at positions 376 and 412 respectively [15,16]. Here, we used the same length of the cytoplasmic fragment of SpoIIE as Lucet *et al.* and confirmed the direct interaction between FtsZ and $SpoIIE_{cyt}$ using sedimentation and light scattering assays, which suggests that the constructs used by Rawlings *et al*., lacked a critical part of the protein for interaction with FtsZ. Our results clearly suggest that the interaction between $SpoIIE_{cyt}$ and FtsZ occurs mostly in the presence of GDP, whereas in the work of Lucet *et al.* direct interaction using gel filtration was found in the

presence of GTP although the interaction was found between SpoIIE and monomers and very short oligomers (3-4 subunits) of FtsZ. It is possible that GTP was hydrolyzed during this the assay as Mg^{2+} was included in the gel filtration buffer, which would explain why FtsZ did not form large structures which should elute much earlier from the column [15]. We did not find evidence for a specific interaction of polymerized FtsZ with Ms-$SpoIIE_{cyt}$. It is possible that the large, 40 kDa MBP tag covers FtsZ binding site on $SpoIIE_{cyt}$ upon oligomerization of $SpoIIE_{cyt}$. Therefore, the binding site is only accessible for FtsZ monomers and short oligomers. We do not exclude the possibility of interaction between FtsZ-GTP and strep-$SpoIIE_{cyt}$ as we noticed a significant increase in the light scattering signal when both proteins were present in the sample with GTP. However, the increase in the light scattering signal had a significant lag phase, which appeared to be related to the moment of the disassembly of FtsZ polymers. Therefore, we hypothesize that $SpoIIE_{cyt}$ preferentially interacts with FtsZ-GDP, possibly by stabiling FtsZ-GDP filaments and thus preventing filament disassembly.

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